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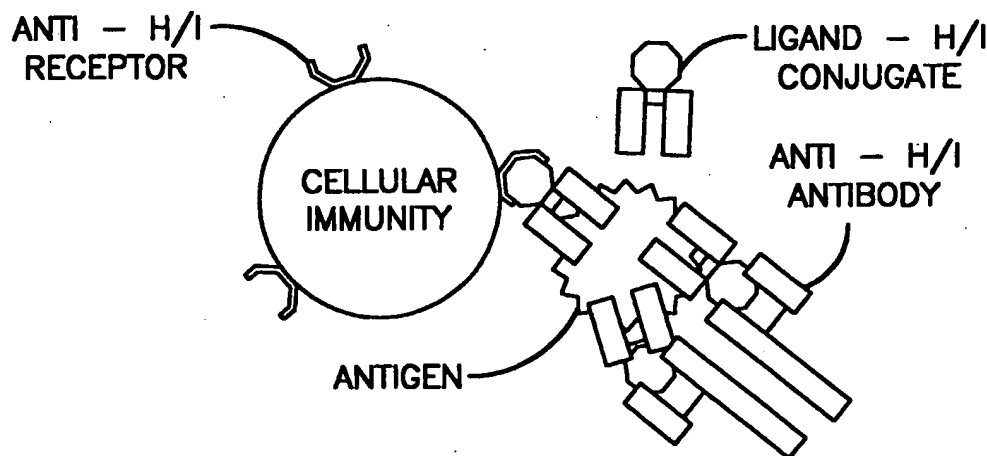
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(54) Title: METHODS FOR CONFERRING ACTIVE/PASSIVE IMMUNOTHERAPY



(57) Abstract: The Active/Passive Immunotherapy of the present invention combines active immunization to a hapten or immunogen (H/I) and passive immunization with the same H/I conjugated to an antigen binding ligand. The prior immunization to the H/I grants active standing immunity to the H/I whereas the antigen binding ligand grants instant passive binding to the target antigen. The ligand - H/I conjugate acts as a "sandwich" molecule to provide binding sites to both the target antigen and immune cells to thereby usurp the active standing immunity for the H/I to passive instant immunity for the pathological target antigen. Particular advantages of the present invention include that chemicals as well as peptides can be used as antigen binding ligand, and the full immune repertoire of humoral and/or cellular immunity can be instantly and selectively mobilized and targeted to a pathological antigen. Thus, the present Active/Passive Immunotherapy can provide a generic defense against the myriad of pathological antigens including microbes and other infectious agents, toxins, as well as autoimmune and cancer antigens.

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## METHODS FOR CONFERRING ACTIVE/PASSIVE IMMUNOTHERAPY

### RELATED APPLICATIONS

The present application claims the benefit of priority from U.S. Patent Application Serial No. 09/429,491 filed October 29, 1999.

### FIELD OF THE INVENTION

The present invention relates to methods for priming human and other mammalian immune systems to thereby confer upon them the ability to react immediately to a wide variety of antigenic substances previously unseen by the immune system.

### BACKGROUND OF INVENTION

The present Active/Passive Immunotherapy (API) invention is designed to combine the advantages of active standing and passive instant immunization. API is accomplished by first giving an active immunization with a hapten and/or immunogen (H/I). The H/I or antigen determinant of said H/I is further conjugated to an antigen binding ligand that can be an antibody, antibody fragment, peptide or chemical capable of binding to a pathological "target" antigen(s). After active standing "sentinel" immunity to said H/I has developed, the subject is administered a passive immunization that comprises the ligand-H/I wherein the ligand grants instant passive binding to the target antigen. The ligand-H/I conjugate acts as a "sandwich" molecule to usurp the active standing immunity for the H/I to passive instant immunity to the pathological target antigen.

The mammalian immune system comprises humoral antibody and cellular immunity to antigenic determinants, "target" antigens or "pathological" antigens. Pathological antigens include infectious agents such as bacteria, viruses and mycological agents, toxins, chemicals, cancer cells and in the case of autoimmunity, an organism's own tissues or portions thereof. The immune system functions to identify, attack and neutralize pathological antigens. Another function of the immune system is to maintain a mammal's tolerance to self-antigens. Both of these functions can be augmented by active or passive immunization. Active

immunization requires prior exposure and therefore sensitization to an antigen and thereby grants "standing" immunity following such exposure.

Typically, exposure to an antigen is by way of vaccination with the antigen or by exposure through infection by a microbe or the breach of epidermal layers caused by injury or infection. After a period of time following exposure to the antigen, typically a few days or weeks, the immune system has processed the antigen to produce antigen-specific cells, and/or antibodies to the antigen. The antigen-specific cells and antibodies to the antigen are the "memory" of the organism's immune system regarding that specific antigen. These antigen-specific antibodies and antigen-specific "memory cells" are thus available immediately should the organism be exposed again to the same antigen.

The antigen-specific memory cells comprise the organism's "cellular" response to the antigen. The antigen-specific antibodies comprise the "humoral" response of the organism. Some antigens produce only a cellular response while others produce only a humoral response. Still other antigens produce both a humoral and a cellular response. Together, the cellular and humoral response components to a specific antigen comprise the "active" or "standing" response of the organism to the antigen. Mammals and other organisms also have "passive" immunity to many antigens. Passive immunity to an antigen can be either innate or provided. The innate passive immunity to many antigens possessed by many organisms is at least partly a result of selective pressures of co-evolution of the organism with many microbes. Typically, mammals have innate passive immunity to hundreds of thousands or millions of antigens. Passive immunity can also be provided, however, by the injection or other administration of antigen-specific cells or antibodies.

Passive immunization grants "instant" immunity by administering antigen specific cells or antibodies. The immune response can select for cellular or humoral immunity for the best mode for neutralization of the target antigen. For example, humoral immunity is generally best for neutralizing toxins and bacteria, and cellular immunity for viral and cancer antigens. The ability of the natural immune response to select primarily either a humoral or cellular, or appropriate combination of humoral and cellular immunity to a pathological antigen is a critical function of the immune response. The lack of effectiveness of many immunotherapies is a direct result of their failure to achieve appropriate activation of cellular and humoral immune responses.

Haptens and immunogens can comprise small synthetic molecules that are not cross reactive with naturally occurring antigen determinants. Haptens can elicit only humoral immune response and are antigenic only when conjugated to a carrier molecule, e.g., peptide or chemical, that is an immunogen. Immunogens elicit both humoral and cellular immunity, but can be selective for cellular immunity. Bifunctional antigens can have both hapten and immunogen determinants. Thus a hapten, immunogen, or bifunctional hapten-immunogen can be selective for either humoral and/or cellular immunity to achieve an optimal balance of humoral and cellular immunity. Vaccines have not been realized for many pathological antigens and it is a critical limitation of both vaccination and passive immunotherapy that often only non-selective or partial humoral or cellular immune responses are achieved. A particular advantage of API is that the full immune repertoire of humoral and/or cellular immunity can be instantly and selectively mobilized to H/I and transferred toward a target pathological antigen(s). Therefore, API could provide a generic defense against the myriad of pathological antigens to include infectious agents, autoimmune and cancer antigens.

Peptide-chemical, chemical-chemical or antibody-chemical conjugates, and immunization to immunogen or hapten-carrier antigenic determinants date back to Landsteiner's work on haptens in 1914, and intact antibody and Fab or Fv regions of antibody have been routinely used to target antigen. Immunotherapies that involve vaccination (Jenner 1796) or passive immunization with antisera (Behring & Kitasato, 1890) are also well known. However, API combines the use of active and passive immunization to provide a substantial improvement over either active or passive immunotherapy used separately. Furthermore, the more recent advances of recombinant DNA, monoclonal antibodies (Kohler & Milstein, 1975), techniques to sequence and synthesize, and methods of increasing the binding affinity of the antigen binding sites of antibodies make API technology much more attractive. Such advances have resulted in the current availability of peptide sequence libraries of the active sites of antibodies that provide an abundant source of API antigen binding ligands. Computer technologies for molecular modeling can also be applied to improve haptens, immunogens, and peptide or chemical antigen binding ligands.

API overcomes a number of problems that are associated with other immunotherapies such as vaccination, anti-sera, monoclonal antibodies, superantigen-ligand, anti-Fc receptor-ligand, and *Staphylococcus aureus* protein A-ligand. These include:

- (1) An intact antibody in anti-sera or monoclonal antibody preparations developed in one species, e.g., horse, mouse, can when administered to another species elicit immunity to the antibody, limiting the clinical use of the antibody. The small size and selective antigenic stimulation by ligand-H/I conjugate should greatly reduce or even eliminate this obstacle.
- (2) The reagents for immunization of a subject to H/I and production of ligand conjugated to H/I can be achieved by known and inexpensive synthesis processes. This allows ligand-H/I to be administered at doses that are compatible with natural concentrations of humoral antibody and levels of cellular immunity.
- (3) The H/I conjugated to the ligand can be a single monovalent antigenic determinant so as not to aggregate either antibody or cellular receptors until the target antigen is engaged.
- (4) Ligands can be designed to achieve the desired valence, specificity and affinity for a given antigen determinant(s), and a specific ligand need not be limited to a peptide or a hapten. Similarly, immunogens may include other types of molecules such as chemicals. This allows chemicals that are used to image or bind to pathological antigens to be used as ligands.
- (5) The method may also help antigen processing, thus establishing immunity to pathogenetic antigenic determinants that normally only weakly simulate immunity.
- (6) The half-life of small ligands, e.g., antibody Fab or Fv, are short compared to intact antibodies. The binding of ligand – H/I conjugates to anti-H/I antibodies or cellular receptors increases ligand half-life.
- (7) Particularly important is that the method can neutralize any antigen that can be bound to a ligand by selectively stimulating a partial or the full immune repertoire to include all antibody classes and cellular immune responses elicited by the H/I.
- (8) It is an important advantage of the invention that a subject need not receive a vaccination for each of the myriad of pathological antigens to which they might need immunity. This foregoes the expenses of developing a multitude of vaccines and the risk of receiving a multitude of immunizations.
- (9) It is an important advantage of the invention that API can be administered as prophylaxis or therapy as a medical countermeasure to an identified pathological antigen or as a broad spectrum antigen binding ligand that could be used to neutralize an entire class of pathogen such as gram negative bacteria.

- (10) It is a particular advantage of the invention that, unlike standard immunization where established immunity is present, withdrawing administration of the ligand H/I conjugate can terminate the API immune response and any toxic side effects.

U.S. Patent 5,189,014 issued on Feb. 23, 1993 to Cowan and entitled "A Method for Treating Cellular Fc Receptor Mediated Immune Disorders" discloses a method of treating immune complex diseases and associated pathological immune regulation using Fc receptors (FcR) or FcR-like bacterial proteins, such as Staphylococcus aureus protein A (SPA). To the best of the inventor's knowledge that patent reports the first immunotherapeutic drug use of a cellular immune receptor, FcR, and a microbial protein (SPA) that "counterfeits" the activity of the receptor. Furthermore, the role of valence, the number of binding sites required for aggregation of antigen or crosslinking of antibody, antigen-antibody immune complexes, ligands and receptors to activation of the immune response are well known in the medical art and were extensively discussed in the patent. The patent also describes binding of SPA to an antigen binding ligand to activate humoral immunity to pathological antigens.

The concept of ligand-hapten conjugations for redirecting humoral immunity to lyse target cells has been previously tested in vitro (Circolo & Borsos, "Lysis of hapten-labeled cells by anti-hapten IgG and complement", J. Immunol. 128, 1118-21, 1996) and proposed in vivo (Shokat & Schultz, "Redirecting the immune response: Ligand-mediated immunogenicity" J. Am. Chem. Soc. 113, 1861-2, 1991). Lussow et al., (SangStat Medical Corp. Menlo Park CA) have recently shown that redirecting circulating antibodies via ligand-hapten conjugates eliminates target cells in vivo (Lussow AR, Buelow R, Fanget L, Peretto S, Gao L, Pouletty P. "Redirecting Circulating Antibodies via Ligand-hapten Conjugates Eliminates Target Cells In vivo", J Immunother Emphasis Tumor Immunol, 19, 257-65, 1996).

The method of anti- T cell ligand-hapten of Lussow et al. selected for only humoral immunity against T cells and they were able to achieve only a  $\leq 50\%$  reduction of circulating T cells (p. 262, top col. 2). Although they attribute this to non-specific binding of anti- T cell ligand, the lack of the cellular response to T cells in their system may have contributed to the failure to more substantially clear T cells from circulation. In any case, the presence of cellular immunity to the circulating T cells may well have overcome this limitation of T cell destruction.

The prior art and the Lussow et al. publication make no mention of the utility of chemical antigen binding ligands to target pathological antigens or immunogens to activate cellular immunity. The present API invention includes the use of chemical ligands and immunogens providing both advantages over concepts beyond the scope of the prior art and the Lussow et al. article. The use of chemical ligands allows chemicals that are routinely used to image and more "generically" bind pathological antigens, such as vital stains, to be used as antigen binding ligands. Furthermore, this improvement will stimulate routine testing of known chemicals and synthesis of new compounds for use as antigen binding ligands. The use of immunogens which, like haptens, can be small chemical molecules such as L-tyrosine-p-azobenzenearsonate (ABA-Tyr), but unlike haptens can select for cellular immunity, gives a distinct advantage over the use of haptens to generate purely humoral immunity.

#### SUMMARY OF THE INVENTION

The present invention, Active/Passive Immunotherapy, combines active standing immunization to a hapten or immunogen and passive instant immunization with an antigen binding ligand for a pathological target antigen, conjugated to the H/I. Prior immunization to the H/I grants active standing immunity whereas the ligand grants instant passive binding to the target antigen. The ligand-H/I conjugate acts as a "sandwich" molecule to usurp the active standing immunity for the H/I to passive instant immunity to the pathological target antigen. In other words, the ligand-DNP conjugate, the ligand-ABA conjugate or the ligand-H/I conjugate functions to provide binding sites of the appropriate types so that presensitized immune cells can bind with the target antigen complex.

A particular advantage of the present invention is that the full immune repertoire of humoral and cellular immunity can be instantly and selectively mobilized and focused toward the target pathological antigen. By combining active with passive immunization, the present invention can achieve either a selective or a full immune repertoire that balances the optimal combination of humoral and cellular immunity to any antigen that can be bound by a ligand. Thus, the present invention provides an immunotherapeutic system that emulates portions of the natural immune response yet offers the previously unknown advantages of a primed

generic immune defense against a myriad of pathological antigens such as infectious agents, toxins, autoimmune and cancer cell antigens.

It is therefore an object of the present invention to provide methods for active and passive immunity which can be modulated selectively and focused on a specific target pathological antigen.

It is a further object of the present invention to provide methods for conferring immediate passive immunity to a specific target pathological antigen.

It is another object of the present invention to provide methods for alleviating the symptoms of diseases caused by microbes and other pathogens as well as methods for ameliorating cancers and autoimmune diseases.

In accordance with this and other objects of the invention, a method is provided for conferring humoral immunization to a target antigen comprising: A) administering a sufficient amount of a hapten-carrier conjugate to a patient for a sufficient length of time to confer upon the patient active standing immunization to the hapten including the proliferation of antibodies to the hapten, and B) administering to the patient a sufficient amount of a hapten-ligand conjugate wherein the ligand of the conjugate has at least one binding site to the target antigen and the hapten of the conjugate is available for binding with the antibodies such that the target antigen becomes complexed with the hapten-ligand conjugate and with the antibodies to the extent that the target antigen undergoes neutralization by the immune cells of the patient.

In some embodiments of the invention, the ligand is an antibody, an antibody fragment, a peptide or a non-peptide chemical capable of binding to the target antigen. In other embodiments, the ligand is not an antibody or antibody fragment but is capable of binding to the target antigen. For example, the ligand can be a chemical used to image or bind to pathological antigens. As one of skill in the art will appreciate, the neutralization typically occurs by processes typical to the mammalian immune system. These processes include antibody mediated complement attack, cellular cytotoxicity and T cell destruction, that is, the phagocytosing of the immune complexes by the patient's immune cells. Thus, as is known regarding the immune response and the processes of antibody mediated



complement activation, cytotoxicity or phagocytosis, and T cell destruction regarding typical antigens, the target antigen in the present invention can similarly be a toxin or a non-peptide chemical, or a portion of a bacteria, virus, mycological agent, cancer cell or the patient's own tissues.

In accordance with yet other embodiments of the invention, a method is provided of conferring immunization to a target antigen comprising: A) administering a sufficient amount of an immunogen or a hapten-immunogen conjugate to a patient for a sufficient length of time to confer upon the patient, i) active standing immunity to the immunogen or hapten including the proliferation of antibodies to the hapten, and ii) active standing immunity to the immunogen including the proliferation of activated immune cells having binding sites for the immunogen, and then, B) administering to the patient a sufficient amount of a hapten-ligand conjugate wherein the ligand of the conjugate has at least one binding site to the target antigen and the hapten or immunogen of the conjugate is available for binding with the binding sites on the activated immune cells such that the target antigen immediately elicits both humoral and cellular responses from the patient to the extent that the target antigen undergoes neutralization by the immune cells of the patient.

In further embodiments of the invention, the immunogen is selected so that administering the hapten-immunogen conjugate also confers upon the patient active standing immunity to the immunogen in the form of both proliferation of antibodies to the hapten and cellular immunity to the immunogen. In some embodiments of the invention, the ligand is an antibody, an antibody fragment, a peptide or a non-peptide chemical capable of binding to the target antigen. In other embodiments, the ligand is not an antibody or antibody fragment but is capable of binding to the target antigen. For example, the ligand can be a chemical used to image or bind to pathological antigens.

The present methods can be used against many different target antigens, for example, where the target antigen is a toxin or a non-peptide chemical, or a portion of a bacteria, virus, mycological agent, cancer cell or the patient's own tissues. Moreover, the present methods can be used with many immunogens including, among others, where the immunogen is a toxin or a non-peptide chemical, or a portion of a bacteria, virus, mycological agent, cancer cell or the patient's own tissues.

Immunogens of the present invention can be small synthetic molecules that are not cross-reactive with naturally occurring antigen determinants. In addition, an immunogen can be selected such that it elicits both humoral and cellular responses to the target antigen or such that the immunogen elicits only a cellular response to the target antigen. In some embodiments of the invention, the ligand is an antibody, an antibody fragment, a peptide or a non-peptide chemical capable of binding to the target antigen. In other embodiments, the ligand is not an antibody or antibody fragment but is capable of binding to the target antigen. For example, the ligand can be a chemical used to image or bind to pathological antigens. As one of skill in the art will appreciate, the neutralization typically occurs by the phagocytosing of the immune complexes by the patient's immune cells.

#### BRIEF DESCRIPTION OF THE DRAWING

Figure 1 schematically shows a hapten or immunogen {"H/I"}, antigen binding ligand ("Ligand") and their combination to form a "ligand-H/I conjugate" moiety useful in the present methods.

Figure 2 schematically shows a Ligand-H/I conjugate according to the invention bound both to an anti-H/I Receptor on an immune system cell and to a target antigen. The target antigen is shown also bound by an Anti-H/I Antibody specific to the target antigen.

Figure 3 schematically shows the molecular formula of an exemplary hapten, DNP, coupled to an exemplary immunogen, ABA-Tyr by means of a covalently bound spacer molecule.

Figure 4 schematically shows the hapten DNP coupled to a ligand and the immunogen ABA-Tyr coupled to a ligand.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention can be understood with reference to the following examples and with reference to Figures 1 – 4, schematic diagrams depicting a hapten or immunogen {"H/I"}, antigen binding ligand ("Ligand") and their combination to form "ligand-H/I

conjugate" moieties used in the present methods of API to bind to, and neutralize, a target antigen.

Haptens are generally peptides or chemicals of small molecular weight (MW), e.g., about 100 to about 1000 daltons MW. Immunization with a hapten bound to an immunogenic protein or a non-peptide chemical immunogen carrier molecule produces a humoral (antibody) immune response. Immunogens can be large macromolecules, however, for use as reagents for practicing the methods of the present invention. Immunogens that are peptides or chemicals of small molecular weight are most suitable.

Immunogens can produce humoral immunity or be selective for cellular immunity alone. Antigen binding ligands can be intact antibodies, Fab2, Fab, Fv regions antibody fragments, peptides or, also like haptens and immunogens, may be chemicals that selectively bind pathological antigens. The immunological art is replete with descriptions of a multitude of peptide and chemical molecules that are haptens, immunogens and antigen binding ligands. Methods for immunization with haptens or immunogens and conjugation of chemicals and peptides to form ligand-H/I conjugates are also well known in the field. Therefore, as one of skill in the art will recognize, the present invention allows for numerous combinations of specific haptens, immunogens and antigen binding ligands. Thus, the following description and examples are exemplary and not limiting.

#### EXAMPLE 1

Active/Passive Immunotherapy (API) comprises combining active immunization to hapten or immunogen (H/I) and passive immunization with an antigen binding ligand- H/I conjugates to transfer active standing immunity from the H/I to a pathological target antigen. Figure 1 shows schematically a hapten or immunogen {"H/I"}, antigen binding ligand ("Ligand") and their combination to form a "ligand-H/I conjugate" moiety useful in the present methods.

#### EXAMPLE 2

Active/Passive Immunotherapy (API) usurps antigen specific cellular and humoral immunity from H/I to pathological antigen. Figure 2 shows schematically a ligand-H/I conjugate according to the invention bound both to an anti-H/I receptor on an immune system cell and to a target antigen. The target antigen is shown also bound by an anti-H/I antibody specific to the target antigen.

### EXAMPLE 3

Haptens such as dinitrophenyl (DNP) coupled to a carrier molecule can stimulate humoral antibody to DNP; whereas immunogens such as L-tyrosine-p-azobenzeneearsonate (ABA-Tyr) activate cellular immunity. A bifunctional hapten – spacer – immunogen, such as dinitrophenyl-6-aminocaproyl- L-tyrosine-p-azobenzeneearsonate (DNP-ABA-Tyr), can induce antibodies specific for the DNP hapten determinant and cellular immunity to the ABA-Tyr immunogen determinant.

Figure 3 shows one species according to the present invention comprising three moieties. An exemplary hapten, dinitrophenyl (DNP), and an exemplary immunogen, L-tyrosine-p-azobenzeneearsonate (ABA-Tyr), are shown connected by a spacer molecule to form an exemplary bifunctional hapten-immunogen DNP-ABA-Tyr. Immunization with the hapten-DNP-carrier causes a humoral anti-DNP antibody immune response, that is, the production of anti-DNP antibodies. Immunization with the immunogen ABA causes an anti-ABA cellular immune response, that is, the production of immune cells having ABA receptors. Immunization with the bifunctional hapten-immunogen DNP-ABA-Tyr causes both an anti-DNP humoral and anti-ABA cellular response.

### EXAMPLE 4

The hapten DNP can be coupled to a ligand and the immunogen ABA-Tyr can be coupled to a ligand. Active/Passive Immunotherapy can be induced in a subject that has been immunized to a bifunctional hapten-spacer-immunogen such as DNP-ABA-Tyr to achieve an optimal balance of humoral and/or cellular immunity to a target antigen. A subject administered an antigen binding ligand conjugated to DNP would gain (humoral) antibody-mediated immunity. On the other hand, a subject administered a ligand-ABA-Tyr conjugate would gain cellular immunity to the ligand's target antigen. Thus, a subject administered

both ligand-hapten and ligand-immunogen achieves both humoral and cellular immunity to the target antigen.

Figure 4 shows DNP and ABA conjugated to an antigen binding ligand. The ligand-DNP

conjugate, ligand-ABA conjugate or ligand-H/I conjugate acts as a "sandwich" molecule to usurp the active standing immunity for the H/I to passive immunity to the pathological target antigen. In other words, the ligand-DNP conjugate, ligand-ABA conjugate or ligand-H/I conjugate functions to provide binding sites of the appropriate types so that presensitized immune cells can bind with the target antigen complex. A particular advantage of the present method is that the full immune repertoire of humoral and/or cellular immunity can be instantly and selectively mobilized and focused toward the target pathological antigen. The ligand-DNP selects for humoral immunity; whereas, the ligand-ABA selects for cellular immunity. By combining active immunization to H/I and passive immunization to ligand-H/I API can achieve either a selective humoral or cellular, or a full immune repertoire that balances the optimal humoral and/or cellular immunity to any particular antigen that can be bound by an antigen binding ligand.

#### EXAMPLE 5

Testing of API immunogen mediated cellular immune response can be achieved using known methods described in the medical arts as follows. Reagents and Preparation: The toxin ricin is used as a prototypic antigen (inactivated and bound to tanned sheep red blood cells [SRBC] by glutaraldehyde). Ricin and monoclonal antibody to ricin serve as a prototypic antigen binding ligand. The anti-ricin antibodies are cleaved by the protease pepsin to Fab2 fragments and isolated for use as antigen binding ligand. The anti-ricin ligand is conjugated to the chemical immunogen L-tyrosine-p-azobenzenearsonate (ABA-Tyr), that is a prototypic immunogen. A standard model for foot pad delayed hypersensitivity (DH) is used to test for cellular immunogen mediated API, in ABA sensitized mice. The prototypic reagents, i.e., antigen, antigen binding ligand and immunogen, are exemplary and not limiting. For example, a chemical antigen binding ligand could be substituted for the anti-ricin antibody.

The reagents listed below are incubated in vitro and washed to remove unbound reagents.

(1) [SRBC]

(4) [Ricin-SRBC + anti-Ricin-ABA]

(2) [Ricin-SRBC]  
animals]

(5) [Ricin-SRBC, given to ricin sensitized

3) [Ricin-SRBC + anti-Ricin]

Reagents (1,2,3) are negative controls, (4) is an API test sample and (5) is a positive control. Reagents (1,2,3,4,5) are passively administered to the hind foot pad of mice sensitized to ABA and also sensitized to ricin in (5) positive control. Twenty four hour post challenge animals are sacrificed and control (left) and test (right) foot pads harvested and weighed. The ratio of the weight of the test foot pad divided by the weight of the control foot pad is determined. Foot pad swelling that yields a ratio, e.g.  $>2$ , indicates a positive delayed hypersensitivity reaction. Five animals are tested in each group with positive control (5) Ricin-SRBC in ricin sensitized animals pretested to determine adequate sensitization to ABA, viability of DH assay and [C] (concentration) of ABA challenge to elicit a good DH response. If DH to ABA is mediated by API, then positive foot pad ratios are expected in (4) API test animals and (5) positive control animals but not in (1-3) control animals. If DH is present as measured by foot pad weight ratio, this will be confirmed in another group of 5 animals by histopathology studies at 24 hours after injection of reagents.

#### EXAMPLE 6

Testing of API hapten mediated humoral immune response can be achieved using known methods described in the medical arts as follows. Reagents and Preparation: In this example, the toxin ricin is used as a prototypic antigen (inactivated and bound to tanned sheep red blood cells [SRBC] by glutaraldehyde). The anti-ricin antibodies are cleaved by the protease pepsin to Fab2 fragments and isolated, to be used as antigen binding ligand that is a prototype for a chemical ligand. The anti-ricin ABA are then conjugated to the chemical

haptens DNP (dinitrophenol). Rabbit anti-DNP antisera is commercially available. Standard C' (complement) and ADCC (antibody dependent cellular cytotoxicity) assays and the passive cutaneous Arthus reaction are used as prototypic models for testing humoral chemical ligand-API.

The reagents listed below are incubated in vitro and washed to remove unbound reagents.

- |                              |  |
|------------------------------|--|
| (1) [SRBC]                   | (4) [Ricin-SRBC + anti-Ricin-DNP]          |
| (2) [Ricin-SRBC]             | (5) [Ricin-SRBC + intact anti-Ricin (Fc+)] |
| 3) [Ricin-SRBC + anti-Ricin] |  |

Reagents (1-5) are incubated with rabbit anti-DNP antibody in the presence of mouse sera, heat inactivated mouse sera (56° C, 30 min) or splenic leukocytes to assay for complement and ADCC mediated lysis of SRBC (hemoglobin release). The optimum M [C] (molar concentration) of intact anti-ricin (Fc+) for lysis of Ricin-SRBC in the C' and ADCC assays serves as a guide for selection of M [C] of anti-ricin (Fab). If complement and ADCC mediated lysis of SRBC is by API, the lysis will be apparent in the (4) test sample and (5) positive control, and not in control samples (1-3). The optimum [C] of reagents in the in vitro experiments serves as a guide or starting point for determining the optimum [C] of reagents in vivo.

Reagents (1,2,3) negative controls and (4) API test sample are incubated with anti-DNP antibody, washed to remove soluble reagents and passively administered to mice subcutaneous (sc). One hour post challenge animals are infused with Evans blue iv, and, at 2 hours, the animals are sacrificed and checked to determine if a cutaneous Arthus reaction is present, as measured by infusion of dye into the skin. Four animals are tested in each group and each animal receives four sc test injections of different [C] (selected from results of in vitro studies) of reagents (1,2,3) as negative controls, and (5) as positive control. The optimum M [C] of the positive control (5) [Ricin-SRBC + intact anti-Ricin (Fc+)] that produces an Arthus reaction serves as a guide for selection of M [C] of API test reagents (4) [anti-Ricin (Fab)-DNP + Ricin-SRBC + Rabbit anti-DNP] to test the API hypothesis. Groups

of four animals are tested with different [C] of API test reagents (4). If "immediate" humoral immunity to DNP is mediated by API, then infusion of Evans blue is much more apparent at the (4) API test site and (5) positive control than at (1-3) control site. If a cutaneous Arthus reaction is present, as measured by infusion of dye into the skin, this will be confirmed in another group of 4 animals by histopathology studies at 8 hours after injection of reagents.

While the invention has been described with reference to certain preferred embodiments, numerous changes, alterations and modifications to the described embodiments are possible without departing from the spirit and scope of invention as defined in the appended claims, and equivalents thereof.



What is claimed is:

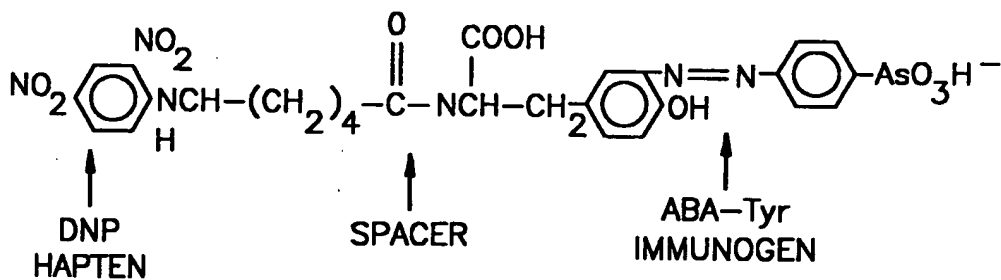
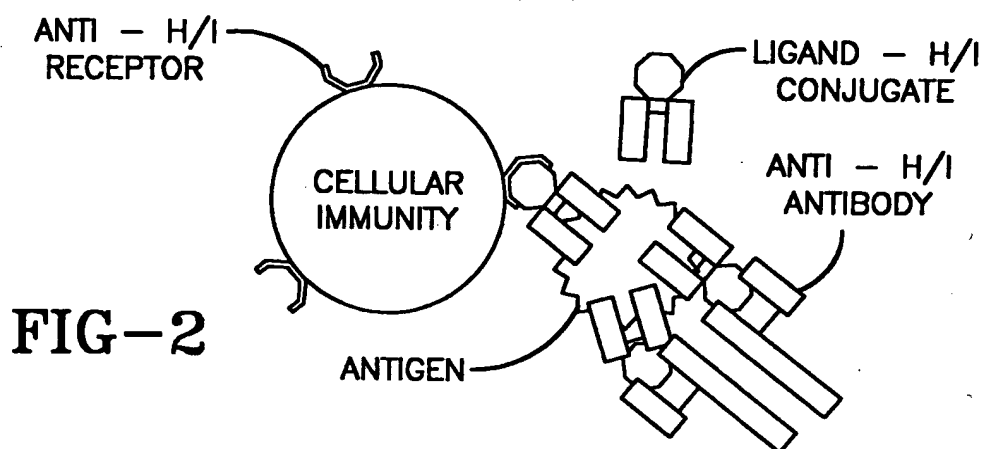
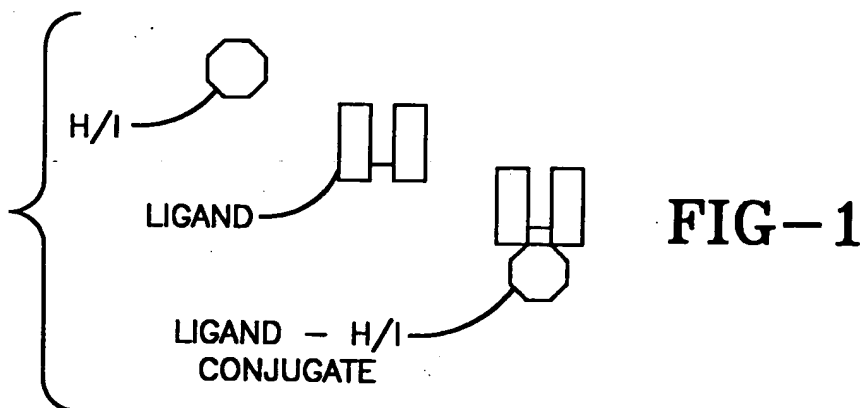
1. A method of conferring humoral immunization to a target antigen comprising:
  - A) administering a sufficient amount of a hapten-carrier conjugate to a patient for a sufficient length of time to confer upon said patient active standing immunization to said hapten including the proliferation of antibodies to the hapten; and
  - B) administering to the patient a sufficient amount of a hapten-ligand conjugatewherein said ligand of said conjugate has at least one binding site to said target antigen and said hapten of said conjugate is available for binding with said antibodies, such that said target antigen becomes complexed with said hapten-ligand conjugate and with said antibodies to the extent that said target antigen undergoes neutralization by the immune cells of said patient.
2. The method of claim 1, wherein said ligand is an antibody, an antibody fragment, a peptide or a non-peptide chemical capable of binding to said target antigen.
3. The method of claim 1, wherein said ligand is not an antibody or antibody fragment but is capable of binding to said target antigen.
4. The method of claim 1, wherein said ligand is a chemical used to image or bind to pathological antigens.
5. The method of claim 1, wherein said neutralization occurs by one or more actions from the group consisting of antibody-mediated complement activation, cellular toxicity and phagocytosis of said immune complexes by said patient's immune cells.
6. The method of claim 1, wherein said target antigen is a toxin or a non-peptide chemical, or a portion of a bacteria, virus, mycological agent, cancer cell or the patient's own tissues.

7. A method of conferring immunization to a target antigen comprising:
- A) administering a sufficient amount of an immunogen or a hapten-immunogen conjugate to a patient for a sufficient length of time to confer upon said patient
    - i) active standing immunity to said immunogen or hapten including the proliferation of antibodies to the hapten, and
    - ii) active standing immunity to said immunogen including the proliferation of activated immune cells having binding sites for said immunogen, and then,
  - B) administering to said patient a sufficient amount of a hapten-ligand conjugate
- wherein said ligand of said conjugate has at least one binding site to said target antigen and said hapten or immunogen of said conjugate is available for binding with the binding sites on said activated immune cells,
- such that said target antigen immediately elicits both humoral and cellular responses from said patient to the extent that said target antigen undergoes neutralization by the immune cells of said patient.
8. The method of claim 7, wherein said immunogen is selected so that administering said hapten-immunogen conjugate also confers upon said patient
- iii) active standing immunity to said immunogen in the form of proliferation of antibodies to said hapten and cellular immunity to said immunogen.
9. The method of claim 7, wherein said target antigen is a toxin or a non-peptide chemical, or a portion of a bacteria, virus, mycological agent, cancer cell or the patient's own tissues.
10. The method of claim 7, wherein said immunogen is a toxin or a non-peptide chemical, or a portion of a bacteria, virus, mycological agent, cancer cell or the patient's own tissues.
11. The method of claim 7, wherein said immunogen comprises a small synthetic molecule that is not cross-reactive with naturally occurring antigen determinants.

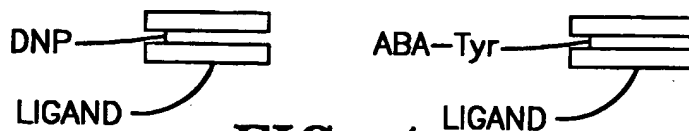
12. The method of claim 7, wherein said immunogen elicits both humoral and cellular responses to said target antigen.
13. The method of claim 7, wherein said immunogen elicits only a cellular response to said target antigen.
14. The method of claim 7, wherein said ligand is an antibody, an antibody fragment, a peptide or a non-peptide chemical capable of binding to said target antigen.
15. The method of claim 7, wherein said ligand is not an antibody or antibody fragment but is capable of binding to said target antigen.
16. The method of claim 7, wherein said neutralization occurs by one or more actions from the group consisting of antibody-mediated complement activation, cellular toxicity and phagocytosis of said immune complexes by said patient's immune cells.
17. The method of claim 7, wherein said ligand is a chemical used to image or bind to pathological antigens.
18. The method of claim 7, wherein said immunogen is a hapten bound to an immunogenic protein, a non-peptide immunogen bound to a carrier molecule, a large macromolecule; or a peptide, or chemical of molecular weight of at least about 500 daltons and less than about 100,000 daltons.
19. The method of claim 7, wherein said hapten is dinitrophenyl and said immunogen is L-tyrosine-p-azobenzenearsonate and wherein said hapten is bound to said immunogen by a spacer molecule of from about 500 to about 10,000 daltons MW.
20. The method of claim 7, wherein said hapten of said hapten-ligand conjugate is dinitrophenyl. and said immunogen is L-tyrosine-p-azobenzenearsonate and wherein said hapten is bound to said immunogen by a spacer molecule of from about 500 to about 10,000 daltons MW.

21. The method of claim 7, wherein said immunogen of said immunogen-ligand conjugate is L-tyrosine-p-azobenzenearsonate.

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**FIG-3**



**FIG-4**

# INTERNATIONAL SEARCH REPORT

Inte. Jonal Application No

PCT/US 00/01112

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K39/385 A61K39/00 A61P39/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

MEDLINE, CANCERLIT, AIDSLINE, LIFESCIENCES, EMBASE, CHEM ABS Data, SCISEARCH, EPO-Internal, BIOSIS, WPI Data, PAJ

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MOOLTEN F.L. ET AL: "Antibodies conjugated to potent cytotoxins as specific antitumor agents." IMMUNOLOGICAL REVIEWS, (1982) VOL. 62/- (47-73). CODEN: IMRED2, XP000915030 page 52, line 4 -page 53, line 13 figure 2  -/-	1,2,5,6

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
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Date of the actual completion of the international search

29 June 2000

Date of mailing of the international search report

08/08/2000

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Covone, M

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/01112

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>LUSSOW A R ET AL: "TARGETING OF ANTIHAPTEN ANTIBODIES TO ACTIVATED T CELLS VIA AN IL-2-HAPTEN CONJUGATE PROLONGS CARDIAC GRAFT SURVIVAL" TRANSPLANTATION,US,WILLIAMS AND WILKINS, BALTIMORE, MD, vol. 62, no. 12, 27 December 1996 (1996-12-27), pages 1703-1708, XP002050537 ISSN: 0041-1337 page 1704, left-hand column, paragraph 4 page 1705, right-hand column, paragraph 2 -page 1706, right-hand column, paragraph 1 page 1706, right-hand column, line 30-37 page 1707, left-hand column, paragraph 3</p>	1,3,5,7, 8,11-13, 15,16,18
X	<p>WO 97 37690 A (SANGSTAT MEDICAL CORP) 16 October 1997 (1997-10-16)</p> <p>example 5 page 2, line 24-30</p>	1,3,5,7, 8,11-13, 15,16,18
A	<p>LEMLEY P V ET AL: "Mice are actively immunized after passive monoclonal antibody prophylaxis and ricin toxin challenge." IMMUNOLOGY, (1992 JUL) 76 (3) 511-3. , XP000915036 page 511, left-hand column, line 6 -right-hand column, line 1 page 513, left-hand column, line 1 -right-hand column, line 6</p>	1-21
A	<p>WO 98 22141 A (SANGSTAT MEDICAL CORP) 28 May 1998 (1998-05-28) claims</p>	1-21
A	<p>WO 98 08875 A (BOHLEN HERIBERT ;VIVA DIAGNOSTIKA DIAGNOSTISCHE (DE)) 5 March 1998 (1998-03-05) page 81, line 13-27 page 8, line 19 -page 9, line 13 figure 1</p> <p style="text-align: center;">-/--</p>	1-21

# INTERNATIONAL SEARCH REPORT

Inte. Jona! Application No

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>DATABASE EMBASE 'Online!  ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM,  NL;  MANZKE O. ET AL: "CD3X anti-nitrophenyl  bispecific diabodies: Universal  immunotherapeutic tools for retargeting T  cells to tumors."  retrieved from STN  Database accession no. 1999266437  XP002141435  abstract  &amp; INTERNATIONAL JOURNAL OF CANCER, (1999)  84/5 (700-708). ,</p>	1-21



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Information on patent family members

International Application No

PCT/US 00/01112

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